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QUANTITATION OF CHLORDIAZEPOXIDE AND ITS METABOLITES IN BIOLOGICAL FLUIDS BY THIN-LAYER CHROMATOGRAPHY

JOHN L. STRAUGHAN*, WILLIAM F. CATHCART-RAKE**, DON W. SHOEMAN and DANIEL L. AZARNOFF***

Clinical Pharmacology and Toxicology Center, Departments of Medicine and Pharmacology, University of Kansas Medical Center, College of Health Sciences and Hospital, Kansas City, Kan. 66103 (U.S.A.)

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SUMMARY

Chlordiazepoxide and its 4 major metabolites were assayed after separation by thin-layer chromatography following extraction from biological fluids. The compounds become intensely fluorescent in the presence of red, fuming nitric acid. The resulting compounds are quantitated with a spectrodensitometer with a fluorescent attachment. The sensitivity varies between 0.05 and 0.1 μg. The coefficient of variation is 1.4% for assays in urine and 6.4% in serum.

INTRODUCTION

Although the benzodiazepines have been studied extensively, many gaps still exist in our knowledge of these agents. As new benzodiazepines become available, research on the earlier compounds becomes somewhat neglected. Chlordiazepoxide (CDX) was the first widely used benzodiazepine and continues to be extensively prescribed; yet, information has only recently become available regarding plasma concentrations of the parent compound and several of its active metabolites after administration of "therapeutic" doses [1, 2]. The biotransformation of chlordiazepoxide has been reviewed by Schwartz [3]. Four major metabolites have been identified in man: desmethylchlor-

*Merck International Fellow in Clinical Pharmacology.
**U.S. Public Health Service postdoctoral trainee in Clinical Pharmacology (GM 07041).
***To whom reprint requests should be sent.

diazepoxide (DM-CDX), demoxepam (DMX), desmethyldiazepam (DM-DZM) and oxazepam (OZM) (Fig. 1).

Spectrofluorometric and gas-chromatographic methods for the quantitation of CDX and its metabolites have been described [4—8]. These methods are not entirely satisfactory since they are often time consuming, do not assay all metabolites, are often affected by interfering substances, and may have inadequate sensitivity. Recently, Strojny et al. [9] reported a method for determination of CDX and three of its major metabolites by spectrophotodensitometry after thin-layer chromatographic (TLC) separation; a fourth metabolite of CDX, demoxepam, was determined by spectrofluorometry after selective extraction. We report here an accurate, precise and relatively simple method using quantitative TLC for the simultaneous assay of CDX and all four major metabolites in biological fluids.

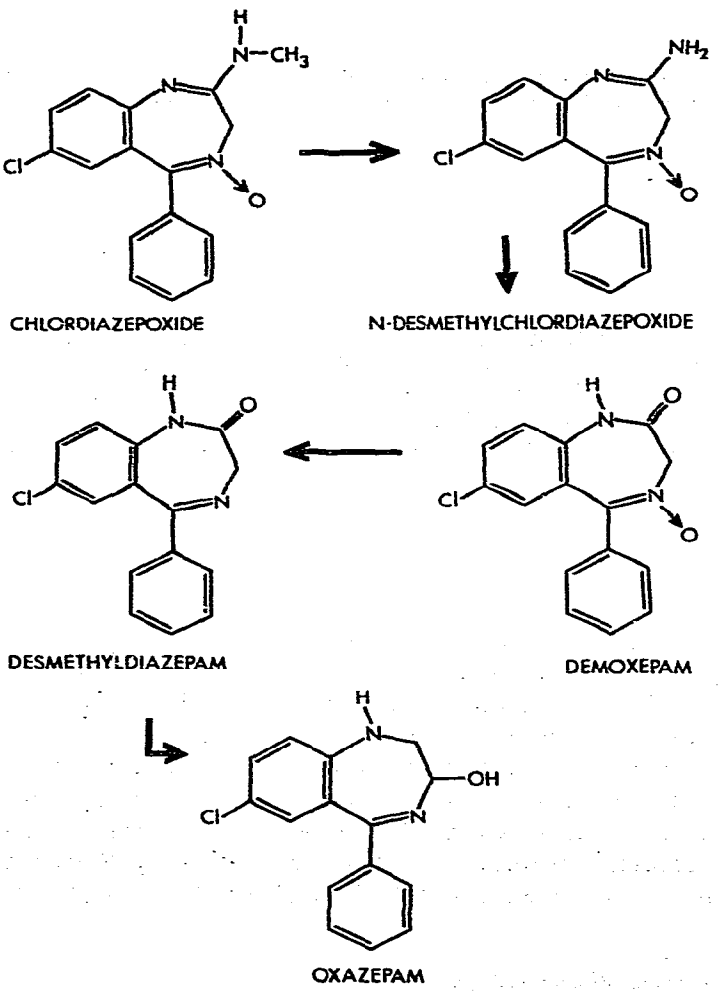

Fig. 1. Major routes of chlordiazepoxide biotransformation in man.

MATERIAL AND METHODS

Extraction from serum

Freshly drawn blood is rapidly centrifuged, preferably in the cold, and the serum stored at −20° until the assay is performed. A 2-ml volume of serum is placed in a 15 ml glass test tube and 0.5 μg prazepam in 5 μl methanol is added as an internal standard. Saturated sodium borate solution (4 ml; pH 9.4) is added to the serum and mixed well. The serum is then extracted three times with 4 ml diethyl ether. After each addition of ether the tube is shaken on a reciprocal shaker at low speed for 10 min, then centrifuged for 5 min in the cold to separate the two phases. The ether extracts are pooled and evaporated to dryness on a constant temperature block at 40° under a stream of nitrogen. The residue is quantitatively transferred to a 10 × 75 mm stoppered glass tube using small volumes of ether and again evaporated to dryness.

Extraction from urine

To assay for unconjugated drugs in the urine, saturated sodium borate solution (5 ml) is added to 10 ml urine and mixed well. Prazepam (0.5 μg) is also added to each urine aliquot. The urine is extracted three times with 5 ml diethyl ether and concentrated following the same procedure outlined above for serum.

Separation of the benzodiazepines

Quanta-Gram LQD, 20 × 20 cm TLC plates scored at 1-cm intervals (Quantum, Fairfield, N.J., U.S.A.) have proved to be very satisfactory for separation and subsequent quantitation of the benzodiazepines. It was not found advantageous to pre-activate these plates. It is not advisable to use the first and last channels of the plates as they tend to run less uniformly than the other channels.

The residue from the ether extraction is dissolved in 100 μl absolute methanol and a 50-μl sample is applied to the spotting area of the TLC plate. A mixture of CDX and its metabolites for use as standards is prepared by dissolving 0.1 mg of each in 1.0 ml absolute methanol. Blank serum or urine is spiked with 0.2, 0.4, 0.8, 1.0 and 2.0 μg of each of the five benzodiazepines to be assayed along with 0.5 μg prazepam. The benzodiazepines are then extracted, redissolved in methanol, and applied to individual channels on the same TLC plate as the unknowns. Fresh standard solutions should be prepared at least every 4 weeks, stored at −20° and protected from light. Standdards should be run on every plate, as no two plates behave identically. The spots are dried in a stream of nitrogen after application to the plate.

Of the many developing solvent mixtures examined, the following was found to give good separation of CDX, its four major metabolites, and prazepam, the internal standard: acetone—dioxane—isopropanol—*n*-heptane—toluene—hexane (15:15:30:30:30:25, v/v) at 22°. Solvents of nanograde quality were used whenever available. The developing mixture must be prepared fresh daily in order to obtain reproducible separation of the benzodiazepines. The TLC tank is lined with filter paper saturated with the solvent mixture

TABLE I

R_F VALUES FOR CHLORDIAZEPOXIDE AND 4 OF ITS METABOLITES, AND FOR PRAZEPAM

Substance	R_F
Prazepam*	0.64
Desmethyldiazepam	0.58
Chlordiazepoxide	0.48
Demoxepam	0.40
Desmethylchlordiazepoxide	0.31
Oxazepam	0.23

*Internal standard.

and the system allowed to equilibrate for 30 min. After placing the TLC plate in the tank, the solvent front is allowed to migrate 16 cm from the baseline (50—60 min). This system separated the areas of maximum fluorescence of each of the compounds by at least 1 cm. Representative R_F values obtained are listed in Table I.

Quantitation of the chromatogram

When exposed to short wave-length ultra-violet light, the benzodiazepines fluoresced to a slight and variable extent. The plate is placed for 45—50 min in a glass tank containing a small beaker of red, fuming nitric acid. This procedure increases the fluorescence of the spots considerably. The plate is removed from the tank, left under a fume hood for 30 min, and then placed in an oven at 100° for 30 min. The latter further intensifies the fluorescence. After this treatment the fluorescence is stable for at least one week.

To quantitate the benzodiazepines, the plate is then scanned with a Schoeffel SD 3000 spectrodensitometer with SDA 335 fluorescence attachment, coupled to a Schoeffel SDC 300 density computer (Schoeffel, Westwood, N.J., U.S.A.). The light source is passed through a Corning No. 271 filter (Corning, Corning, N.Y., U.S.A.). Maximal fluorescence is obtained with the reflectance mode monochrometer of the SDA unit set at 680 nm. The slit width of the exciting beam is 1 mm and the plate speed 2 in. per min. A black line scored across the chromatogram at the junction of the spotting area and the silica gel provides a useful reference point. This line appears on the chart record as a negative deflection. The distance from this point to the peaks is used to calculate R_F values as a check on the identity of the benzodiazepines. One of the unused lateral channels on the TLC plate may be used to set the baseline for the recorder. For each benzodiazepine a standard curve is constructed from a linear-linear plot of the benzodiazepine—prazepam peak height ratio for each benzodiazepine versus concentration. The peak height ratio of each benzodiazepine in the unknown is compared to its respective standard curve for quantitation.

RESULTS

Recovery of each of the 5 compounds added to blank serum or urine is 80% or greater. A 0.5-μg sample of each of the 5 compounds per 10 ml urine or per 2 ml serum can be assayed with a coefficient of variance between 1.4 and 6.4%. The standard curves for all 5 compounds are linear from at least 0.1 to 2.0 μg (Fig. 2). DM-DZM and DM-CDX can be measured in amounts as low as 0.05 μg. CDX, DMX and OZM are easily quantitated in amounts as low as 0.1 μg.

Attempts were made to hydrolyze the glucuronide conjugates by acid hydrolysis and by addition of large amounts of β-glucuronidase. In either case, the conditions required resulted in some degradation of the benzodiazepines. As a result, the distinct separation after extraction of the unconjugated compounds could no longer be attained.

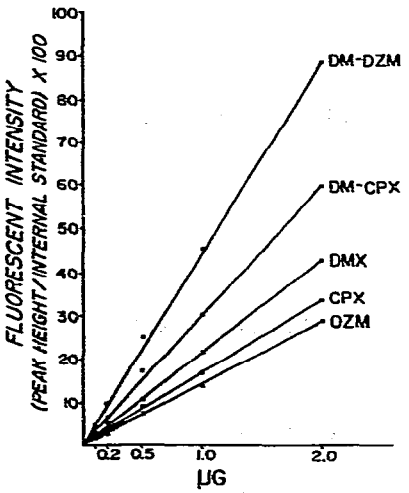

Fig. 2. Standard curves for chlordiazepoxide and each of its major metabolites.

Interfering substances

In order to investigate the possibility of interference produced by drugs (some of which, patients might take concomitantly with CDX) a wide spectrum of drugs was tested in the TLC system (Table II). The specified quantity of each drug was added to blank plasma alone and also with 1.0 μg of each of the 5 benzodiazepines. A band of absorbance (char) appeared just above the fluorescent band of the internal standard, prazepam, in all serum samples analyzed. This band was presumably due to lipids and did not interfere with the fluorescence of prazepam.

Serum levels after single and multiple dose chloridiazepoxide administration

The plasma-level–time relationship of CDX and its 4 metabolites after the administration of a single 50 mg dose of Librium® to a 50 kg female

TABLE II

INTERFERENCE OF DRUGS THAT MIGHT BE ADMINISTERED CONCOMITANTLY WITH CHLORDIAZEPOXIDE

1 = No discernible fluorescence; 2 = absorbance (char), no interference with fluorescence of benzodiazepines

Drug	R_F value	Amount applied (μg)	Interference
Clonazepam	—	2	1
Diazepam	0.59	2	Moderate fluorescence, poor separation from desmethyldiazepam
Flurazepam	—	2	1
Flunitrazepam	—	2	1
Nitrazepam	—	2	1
Temazepam	0.49	2	Strongly fluorescent band overwhelms CDX in this amount
Chlorpromazine	0.13	2	2
Thioridazine	—	2	1
Glutethimide	—	5	1
Meprobamate	—	1.6	1
Amitriptyline	—	2	1
Desipramine	—	2	Narrow band of absorbance just above baseline
Imipramine	0.10	2	2
Nortriptyline	—	2	1
Furosemide	—	1.6	1
d-Propoxyphene	—	2	1
Aspirin	—	2	1
Salicylic acid	—	20	2
Carbamazepine	0.48	5	Absorbance, quenches chlordiazepoxide fluorescence
Phenobarbital	—	40	1
Phenytoin	—	20	1
Quinidine SO_4	—	2	Strongly fluorescent band just above baseline

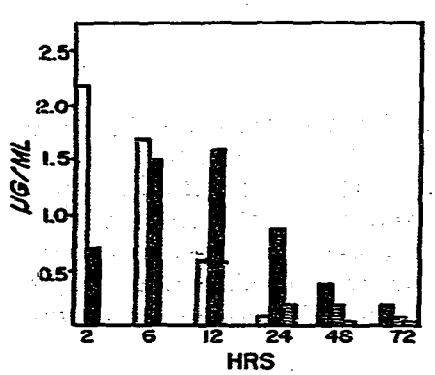

Fig. 3. The plasma concentration of chlordiazepoxide and four metabolites at varying times after administration of a single 50-mg dose of Librium orally (50 kg female). ▨, CDX; ▩, DM-CDX; ▤, DMX; □, DM-DZM.

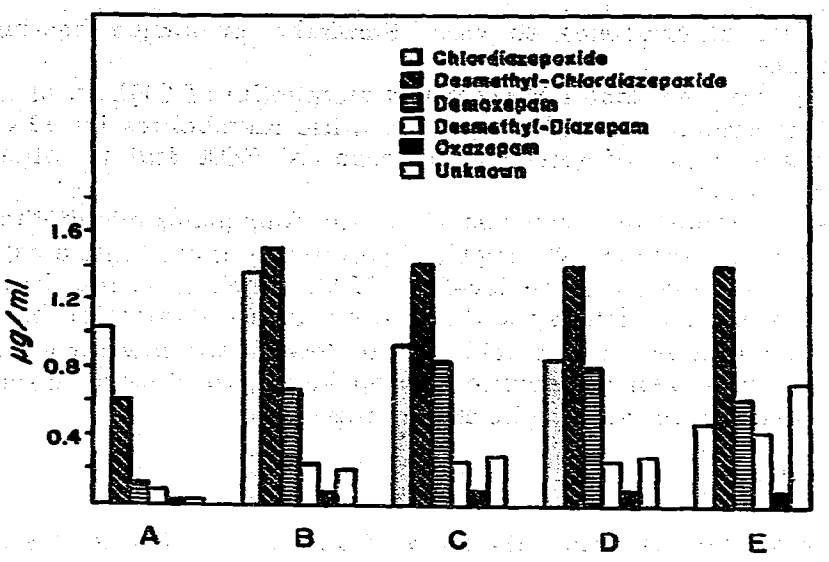

Fig. 4. Plasma concentrations of chlordiazepoxide and four metabolites at varying times after administration of 25 mg Librium every 8 h for 4 days (86 kg male). A = 24 h after first dose; B = 96 h after first dose; C = 12 h after final dose; D = 18 h after final dose; E = 36 h after final dose.

volunteer is shown in Fig. 3. The plasma levels of the 5 benzodiazepines during and after administration of 25 mg of Librium, 3 times a day for 4 days to an 86 kg male volunteer are shown in Fig. 4.

DISCUSSION

Studies on the pharmacokinetics of CDX and its metabolites have been hampered by the lack of specific, sensitive, accurate and rapid assays. We report here an assay which conforms to three of these requirements. Although the assay takes several hours to complete, the method is easily performed and allows 12 samples of blood or urine to be assayed for CDX and 4 metabolites simultaneously, on a single TLC plate. In addition, many other drugs which might be administered concommitantly with CDX do not interfere with the assay for CDX or its metabolites. In our method, elution of the compounds from the TLC plate for quantitation by other means is not required. In contrast to the TLC method of Strojny et al. [9], our method requires neither multiple developments in order to separate lipid materials from the benzodiazepines nor a separate assay method for determination of demoxepam. The precision of quantitation is increased by addition of an internal standard, prazepam. This method can be adapted to the determination of other benzodiazepines by selecting the appropriate developing solvent system.

The use of the borate solution with a pH of 9.4 and gentle shaking of the aqueous ether mixture makes a considerable contribution to ease of extraction by diminishing emulsion formation. The duration of exposure of the chromatogram to the red fuming nitric acid is critical. Excessive exposure

will eventually reduce fluorescence to zero. Similarly, prolonged heating will reduce fluorescence.

Dixon et al. [10] reported that DM-DZM is a metabolite of CDX in man. These investigators separated DM-DZM from the other metabolites by TLC. However, their system does not satisfactorily separate CDX and its other metabolites from each other.

The TLC method we report here can quantitate the four major metabolites in the serum of individuals receiving 25 mg CDX chronically three times a day. Following a single dose, however, the level of OZM, which is at the distal end of the biotransformation pathway and has the shortest elimination half-life of the various metabolites, is detectable but its level is too low for accurate quantitation. An unknown metabolite also appears with chronic dosing (Fig. 4). Studies to identify this metabolite are in progress.

ACKNOWLEDGEMENT

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